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Globulin-specific Proteolytic Activity in Germinating Pumpkin Seeds as Detected by a Fluorescence Assay Method[1]

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ABSTRACT

The proteolytic activities of α-chymotrypsin, trypsin, pepsin, bromelain, and an extract from germinating pumpkin seeds (*Cucurbita moschata*) were determined by their ability to effect the release of 1-anilino-8-naphthalenesulfonate bound to internal hydrophobic sites in intact protein substrates. Casein, glyceraldehyde-3-P dehydrogenase, urease, catalase, pumpkin seed globulin, and bovine serum albumin enhanced the fluorescence of 1-anilino-8-naphthalenesulfonate sufficiently to be used as proteolytic substrates. Chymotrypsin, trypsin, pepsin, and bromelain exhibited activity against all or almost all of the protein substrates. The activity of 1 μg of α-chymotrypsin or trypsin and 100 ng of pepsin could be easily detected by this method of assay within 4 to 5 minutes depending upon the substrate. The enzyme extracted from 3-day germinated pumpkin seeds exhibited strong activity only against pumpkin seed globulin, weak activity against the globulins of squash and cucumber and casein, and no activity against the other protein substrates. Activity against pumpkin globulin was maximal at pH 7.4. When assayed by an increase in ninhydrin-positive products, the enzyme extract from pumpkin seeds also showed strong activity against pumpkin globulin and weak activity against casein. The 1-anilino-8-naphthalenesulfonate-fluorescence method was at least 20 times more sensitive than the ninhydrin method and was 10 to 20 times more rapid.

Germinating seeds and senescing leaves are two types of tissue undergoing rapid protein depletion. The storage proteins of germinating seeds or the total protein of senescing leaves decline greatly in a few days or weeks, respectively, accompanied by an increase in amino acids which are metabolized or transported elsewhere (6, 20, 21). Past attempts in this laboratory and others to detect and determine changes in the proteolytic enzymes responsible for the hydrolysis of tissue protein have beeen disappointing. In many reports, the loss of tissue protein has beeen substantial before any proteolytic activity could be detected (3, 13, 14, 23). The detection of or changes in proteolytic activity had little correlation with the changes in tissue protein levels. Activity often remained constant at a low level both before and during rapid protein decline (13) or else increased after protein had already declined substantially (14).

The conventional method used to determine proteolytic activity in a tissue extract involves incubating the extract with a standard protein such as casein or gelatin, terminating the reaction after a given time, and analyzing the reaction mixture for an increase in ninhydrin-positive, trichloroacetic acid-soluble products (3, 11). Alternatively, the reaction may be followed by titration (2). This general assay method assumes that the proteolytic enzyme(s) in the tissue extract will hydrolyze any protein being specific only in the peptide bond cleaved. In some instances, activity has been demonstrated directly against selected dipeptides (2) or substrates such as TAME[2] or BTEE.

Utilizing a new approach to the problem of analyzing proteolytic activity, we have detected proteolytic activity in germinating pumpkin seeds which exhibits a high degree of specificity towards a specific protein as a substrate.

This paper reports on a new method for the detection of proteolytic activity utilizing the sensitivity of the fluorescence quantum efficiency of the dye ANS. ANS and its analogs have the unique property of being fluorescent in a nonpolar environment or in hydrophobic sites of a protein such as BSA (1, 18). In an aqueous media, ANS is nonfluorescent (7). We have applied this fluorescence property of ANS in a method for detecting proteolytic activity against intact protein substrates. If ANS is initially bound within relatively hydrophobic sites of an intact protein and if a proteolytic enzyme hydrolyzes the protein internally destroying the hydrophobic sites, then with time, ANS will be released from its hydrophobic environment into the aqueous media with a concomitant loss of fluorescence.

This fluorescence method was tested using purified, commercially obtained proteolytic enzymes and was then applied to detect proteolytic activity in extracts from germinating pumpkin seeds.

MATERIALS AND METHODS

The following proteolytic enzymes were obtained from Sigma: α-chymotrypsin (type I, three times crystallized); trysin (type II, twice crystallized); pepsin (twice crystallized and lyophilized); bromelain (grade II, practical); and LAP

[1] This work was supported in part by the Illinois Agriculture Experiment Station.

[2] Abbreviations: TAME: *p*-toluenesulfonyl-L-arginine methyl ester; ANS: 1-anilino-8-naphthalenesulfonate; LAP: leucine amino peptidase; DNS: 1-dimethylaminonaphthalene-5-sulfonylchloride; FITC: fluorescein isothiocyanate; BTEE: benzoyl-L-tyrosine ethyl ester.

(type III-CP, purified). Dilutions of stock solutions of these enzymes were made just prior to assay.

The following commercially purified proteins were used as substrates for the proteolytic assays: α-casein, BSA (type V), catalase, urease (Jack bean), and glyceraldehyde-3-P dehydrogenase. Pumpkin seed globulin was extracted from ungerminated pumpkin seeds according to the method of Bonner (4). Forty cotyledons, with the seed coats and membranes removed, were homogenized in buffered 10% NaCl, pH 7. After remaining at room temperature for 4 hr, the extract was filtered. The filtrate was dialyzed for 24 hr against three changes of distilled water to precipitate the globulin, which was collected by centrifugation and redissolved in buffered 10% NaCl, pH 7. The solution was centrifuged again to remove any nondissolving protein.

In addition, small quantities of pure (by electrophoresis and zonal centrifugation) globulins from pumpkin seed, squash seed, and cucumber seed were obtained from Douglas M. Brown at ULCA. The Mg salt of purified ANS was kindly provided by Dr. Gregorio Weber, University of Illinois.

Enzyme Extraction from Pumpkin Seeds. Pumpkin seeds (*Cucurbita moschata* Poir. vc. Dickinson Field) were placed between layers of moist filter paper and allowed to imbibe water for 2 or 3 days at 25 C, after which the seed coats, membranes, and emerging hypocotyls were removed from the cotyledons. A total of six different buffer systems were tested for efficacy in extracting proteolytic activity from the cotyledons. One buffer system was superior: 0.1 M tris-0.1% Triton X-100, pH 7.4. (The other buffer systems used contained combinations of cysteine, EDTA, NaCl, or sucrose with tris.) All operations were performed at 0 to 4 C. A given number of cotyledons (usually 20 or 30) were homogenized in a VirTis blender in buffer for 1 min and then filtered through Miracloth. The filtrate was centrifuged at 10,000*g* for 20 min. The lipid layer was removed, and the protein in the supernatant was precipitated with 75% saturation ammonium sulfate on ice. The collected protein was redissolved in 50 mM tris, pH 7, and its content was determined by biuret (9). The extracts were assayed for proteolytic activity without further purification.

ANS Fluorescence Enhancement by Protein Binding. An Hitachi Perkin-Elmer MPF-2A speectrofluorimeter was used for fluorescence measurements. The fluorescence of ANS was excited at 370 nm and oserved at 460 nm. To determine whether a given protein enhanced the fluorescence of ANS by providing sufficiently hydrophobic sites for the probe, the relative fluorescence intensity of various concentrations of ANS mixed with a constant concentration of each protein was determined over a wide pH range. At each pH, the quantity of ANS sufficient or in slight excess of complete binding (as determined by no further increase in fluorescence) was determined for each protein substrate.

Fluorescence Assay for Proteolytic Activity. Proteolytic assays were performed using the following concentrations of protein substrates in a reaction volume of 3 ml: casein, 0.33 mg/ml; BSA, 66 μg/ml; catalase, 0.33 mg/ml; urease, 0.17 mg/ml; glyceraldehyde-3-P dehydrogenase, 0.33 mg/ml; and pumpkin globulin, 0.17 mg/ml. The purified proteolytic enzymes were assayed at their pH optimum, as reported in the literature, in a buffer containing any necessary additives such as $CaCl_2$ or $MgCl_2$.

The incubation mixture with buffer, protein substrate, and ANS was equilibrated in the cuvet at approximately 25 C until a steady fluorescence intensity was observed, usually within 2 min. A given quantity of a proteolytic enzyme (100 ng–100 μg) in a volume of 0.1 ml was added, the mixture was shaken, and the recorder was started. The decline in fluorescence intensity was followed for up to 20 min, although 5 min was usually adequate to determine linearity of the reaction rate. Two types of blanks were run with each assay. One blank lacked the proteolytic enzyme and was used to determine background changes in ANS fluorescence with time for the protein substrate. (This blank is important when testing a protein substrate isolated from a tissue extract which could also contain some proteolytic activity.) Another blank lacked the protein substrate and was used to determine ANS binding to the proteolytic enzyme protein at the concentration used. (This blank is important for screening tissue extracts which may contain ANS-binding impurities or impurities having inherent fluorescence or absorption.) The activity of each proteolytic enzyme is reported as the loss of fluorescence in relative units/min·mg enzyme or extract protein.

Ninhydrin Assay for Proteolytic Activity. For two enzyme-substrate systems, proteolytic activity was also assayed by the standard ninhydrin method (11). Assay mixtures (volume 3 ml) were incubated at 37 C. At t_0 and several time intervals thereafter, duplicate reaction samples were terminated by the addition of 2 ml of 15% trichloroacetic acid. Precipitated protein was removed by centrifugation and 0.5 ml of the supernatant was used in the determination of amino acids by the ninhydrin reaction (22). Activity by this method is reported as ΔA_{570}/min·mg protein.

RESULTS

Six proteins were selected for their ability to bind ANS with fluorescence enhancement and for interest as potential proteolytic substrates. Table I gives the molar ratio of ANS-protein for each at pH 7.8. Fluorescence enhancement was usually more pronounced at lower pH. With all six proteins, ANS fluorescence was excited maximally between 350 to 370 nm with peak emission between 460 to 480 nm.

Activity of Purified Proteolytic Enzymes. Figure 1 shows the typical decline in ANS fluorescence when casein is hydrolyzed by increasing quantities of α-chymotrypsin. Within 4 to 5 min, the activity of 1 μg of chymotrypsin could be easily detected. Over longer times (up to 20 min), the activity of 100 ng of chymotrypsin could be easily detected. Time studies longer than 20 min were not performed. Figure 2 shows the linearity of the reaction rate of chymotrypsin versus enzyme quantity for the five substrates tested with chymotrypsin. Activity was proportional to enzyme quantity up to 40 μg in most cases. At pH 7.8, chymotrypsin exhibited greatest activity against casein, followed in order by urease, catalase, glyceraldehyde-3-P dehydrogenase, and BSA.

The activities of the other commercial, purified proteolytic enzymes are summarized in Table II. Within 8 to 10 min at pH 7.8, the activity of 100 ng of trypsin could be detected against casein, which was the most susceptible substrate. No tryptic activity against BSA or catalase could be detected within the limits of trypsin concentration and time period used. (BSA is relatively inert to proteolytic attack above pH 5 because of its tight globular tertiary structure [19].)

Pepsin exhibited greater activity against more substrates than did trypsin or chymotrypsin. Pepsin exhibited its strongest activity against pumpkin seed globulin. Against BSA, pepsin at pH 2.7 exhibited a biphasic reaction rate (Fig. 3). An initial slow loss of ANS fluorescence (rate I) was followed by a faster rate of loss (II). These rates are given in Table I as (I) and (II).

No activity of the exopeptidase LAP could be detected over a 30-min period by this method even when 100 μg of the enzyme was used.

Table I. *Molar Ratios of ANS-Protein for Selected Proteins*

The relative fluorescence intensity for increasing quantities of ANS in the presence of a constant amount of protein in a volume of 3 ml was determined over a wide pH range. ANS fluorescence was excited at 370 nm and observed at 460 nm. The quantity of ANS at which little or no further increase in fluorescence was observed is given as the saturation amount. Values are given for ratios at pH 7.8.

Protein Substrate	Mole Wt	Quantity in 3 ml		ANS at Saturation	ANS-Protein
		mg	*nmoles*	*nmoles*	*molar ratio*
Casein	33,600	1.0	30	32.2	1:1
Urease	480,000	0.5	1	22.6	22:1
Catalase	250,000	1.0	4	12.9	3:1
Glyceraldehyde-3-P dehydrogenase	140,000	1.0	7	29	4:1
BSA	66,000	0.2	3	18	6:1
Pumpkin seed globulin	240,000	0.5	2	45	22:1

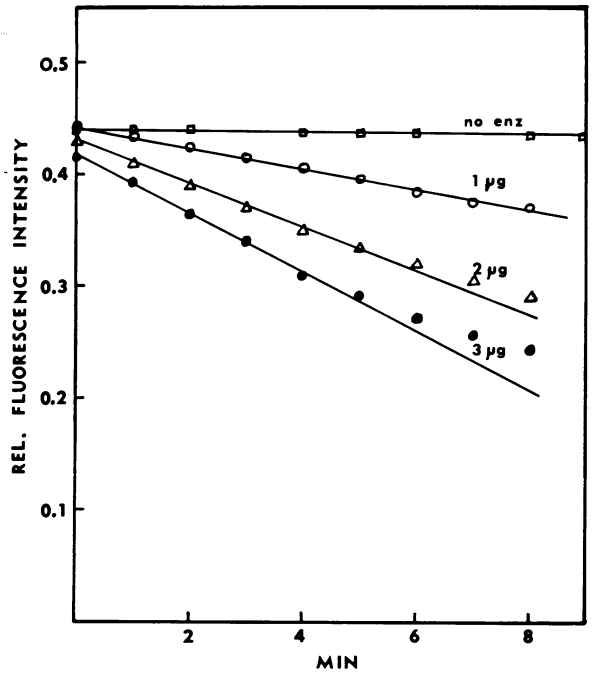

FIG. 1. Loss of ANS fluorescence with time during the hydrolysis of casein by α-chymotrypsin. The assay mixtures contained 7 μg of ANS, 1 mg of casein, 50 mM tris-0.1 M $CaCl_2$, pH 7.8, and the indicated amount of chymotrypsin in a volume of 3 ml.

Proteolytic Activity in Extract from Pumpkin Seeds. The enzyme extract from 3-day germinated pumpkin seeds exhibited strong activity only against pumpkin seed globulin (Fig. 4). The enzyme preparation exhibited some inherent activity (presumably due to globulin present in the extract) above which a weak activity was detected against casein. The activity against casein was approximately 2% as much as that against pumpkin globulin. No activity was observed against catalase, urease, BSA, or glyceraldehyde-3-P dehydrogenase. The activity against pumpkin globulin was maximal at pH 7.4, but was detectable from pH 5.4 to 7.8. The addition of $CaCl_2$ to the assay mixture reduced the activity against globulin.

An enzyme extract from 2-day germinated pumpkin seeds was also tested against several substrates, including the pure plant globulins. The results, summarized in Table III, indicate that the enzyme extract exhibited three to four times greater activity against its own globulin than any other globulin.

An enzyme extract from 2-day germinated pumpkin seeds was also tested for proteolytic activity by the ninhydrin

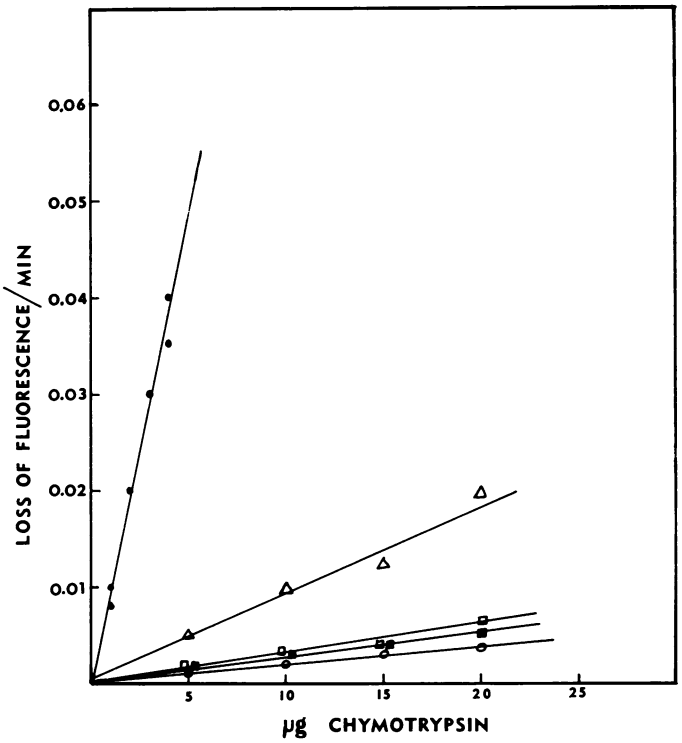

FIG. 2. Activity of chymotrypsin as a function of enzyme concentration. The indicated quantities of enzyme were incubated for up to 20 min with constant amounts of ANS and protein-substrates in a volume of 3 ml. The protein-substrates were casein (●); urease (△); catalase (□); glyceraldehyde-3-P dehydrogenase (■); BSA (○).

Table II. *Average Activity of Several Proteolytic Enzymes Against Intact Protein Substrates Assayed by Decline in ANS Fluorescence*

The proteolytic enzymes were tested for their ability to hydrolyze the given protein substrates and effect the release of ANS bound to the protein substrates resulting in a decline of ANS fluorescence. Each enzyme was assayed at its reported pH optimum. The quantities of protein-substrate and ANS in each assay are given in Table I.

Protein Substrate	Proteolytic Enzymes			
	Chymotrypsin	Trypsin	Pepsin	Bromelain
	Change in relative fluorescence/min·mg enzyme			
Casein	9.0	8.5	5.0	0.2
BSA	0.2	ND[1]	19.0 (I)[2] 49.0 (II)	ND
Catalase	0.3	ND	57.0	ND
Urease	1.0	1.0	7.0	0.3
Glyceraldehyde-3-P dehydrogenase	0.3	0.8		8.0
Pumpkin globulin		2.2	840	0.5

[1] Activity was not detectable.

[2] Designations (I) and (II) refer to the two rates of activity exhibited by pepsin against BSA.

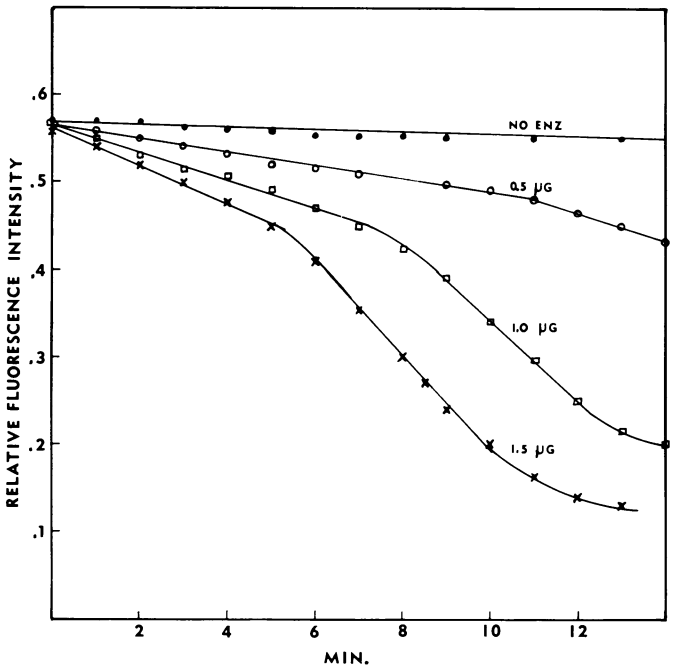

FIG. 3. Biphasic rate of ANS fluorescence decline due to the hydrolysis of BSA by pepsin at pH 2.7.

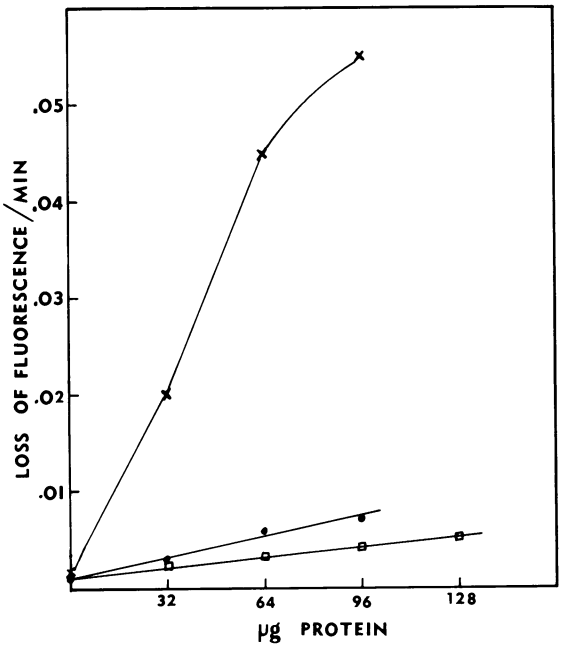

FIG. 4. Proteolytic activity of a extract from 3-day germinated pumpkin seeds as detected by the loss of ANS fluorescence. ANS was bound to pumpkin seed globulin (×) or casein (●). When incubated alone in the presence of ANS, the enzyme extract exhibited a small inherent activity (□). Assays were performed at pH 7.4.

method. These results, given in Figure 5, confirm the results of the fluorimetric assay. No activity against casein was detectable until 30 min after initiation of the reaction.

Table IV gives a comparison of the ninhydrin assay method with the ANS-fluorescence assay method for two systems: pumpkin seed enzyme extract/pumpkin globulin and pepsin/BSA. The ANS-fluorescence method was approximately 20 times more sensitive for the extract proteolytic activity and 4 times more sensitive for the pepsin activity in terms of the change in fluorescence relative to the change in absorption per mg enzyme or extract protein.

DISCUSSION

The loss of fluorescence when ANS is released from the internal hydrophobic sites of a protein requires that the protein be hydrolyzed internally for the most rapid results. The rate of fluorescence loss depends upon how rapidly the protein's hydrophobic regions are affected by the hydrolysis. It would be possible for a proteolytic enzyme to make many cleavages before the hydrophobic regions were affected, in which case the apparent activity against that protein would be low. On the other hand, the proteolytic enzyme might hydrolyze only a few bonds and immediately destroy the hydrophobic regions of the protein. For this reason, a close comparison of the rates of activity against the different substrates should not be made, especially for the purified proteolytic enzymes. This limitation to the technique may be partially overcome by utilizing the

Table III. *Proteolytic Activity of Extract from Pumpkin Seeds*

Pumpkin seeds were germinated for 2 days and then extracted as described in "Materials and Methods." Activity was determined by the decline of ANS fluorescence during the hydrolysis of the given proteins by proteolytic enzymes present in the extract. Activity is given as the loss of fluorescence in relative units/min·mg extract protein.

Substrate	Proteolytic Activity
Dialyzed pumpkin seed globulin	0.21
Pure pumpkin seed globulin	0.18
Pure squash globulin	0.07
Pure cucumber globulin	0.05
Casein	0.005

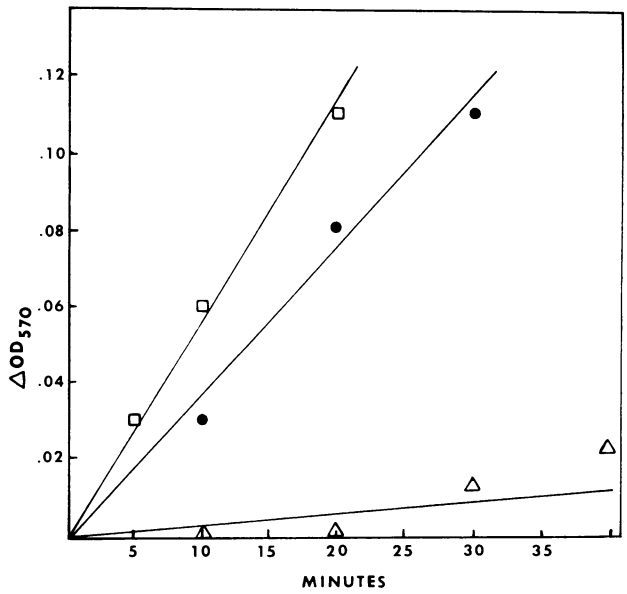

FIG. 5. Time course of pumpkin seed proteolytic activity as determined by the increase in ninhydrin-positive products. Activity was detected against casein with 0.53 mg extract protein (×); against globulin with 0.26 (●) and 0.53 (□) mg of extract protein. Duplicate samples were taken at each point.

sensitive measurements of fluorescence polarization with the fluorescent dyes DNS or FITC covalently bound to the protein substrate (15).

In general, the digestive proteolytic enzymes exhibited activity against most, if not all, of the substrates tested, as would be expected from their *in vivo* function (12). Bromelain also exhibited a wide range of activity. The length of time for the fluorescence assay method and the limits of sensitivity are as good or slightly better than assay techniques using TAME or BTEE as substrates for trypsin and chymotrypsin, respectively. This fluorescence method offers the advantage of detecting the rate of proteolytic activity against a particular intact protein.

Unlike the purified proteolytic enzymes, the proteolytic enzyme(s) extracted from pumpkin seeds exhibited strong activity only against one substrate, pumpkin seed globulin, which is the main storage protein in the cotyledons.

Pumpkin seeds contain 26% protein, of which 93% is globulin (6). An albumin is also present in the cotyledons. During the first three days of germination (the maximaum age of the seeds used in these experiments), pumpkin cotyledons lose about 20% of their protein content. However, little if any significant proteolytic activity using casein hydrolysis was detected until after 6 days, by which time the cotyledons had lost almost 50% of their protein (2, 6).

It appears that the lack of detection of proteolytic activity in germinating pumpkin seeds (and other seed types) is a result of the choice of substrate with which to detect activity. The choice of substrate may be critical. The best substrate would appear to be a protein that is known to disappear or lose activity in the tissue during the time period in question, such as the globulin in the pumpkin seeds.

Undoubtedly, more than one proteolytic enzyme is present in germinating seeds (2, 10, 11, 13). It is possible that one or more proteolytic enzymes, with high specificity for the storage protein initially hydrolyze the protein into smaller units. Subsequently, other proteolytic enzymes complete the hydrolysis. The weak activity detected against casein in this work and earlier reports may be due to these less specific enzymes.

Why does the proteolytic enzyme(s) extracted from the cotyledons exhibit a strong preference for the globulin as a substrate? Pumpkin globulin is rich in arginine (13%); but if the enzyme were preferentially hydrolyzing peptide bonds involving arginine or basic amino acids, then urease (also rich in arginine) should have been attacked also. No activity was detected against urease, although trypsin which hydrolyzes the carboxyl peptide bond of basic amino acids (12) hydrolyzed urease very well. It is possible that the pumpkin seed proteolytic enzyme possesses specificity which depends upon some feature of the globulin tertiary structure.

Little is known about the occurrence and nature of proteolytic enzymes possessing protein specificity. Brush (5) has reported on a proteolytic enzyme with high specificity against insulin. A protein which inactivates nitrate reductase has been isolated (16, 17). Considering the orderly and regulated turnover of proteins in most tissues (8), it seems likely that protein-specific proteases are more common than has been demonstrated, due to lack of methodology and sufficiently pure proteins to be tested as substrates.

As a method for detecting the activity of endopeptidases, this assay technique utilizing ANS fluorescence offers several distinct advantages for detecting protein-specific proteases: activity can be determined rapidly (5–10 min) with no subsequent analysis of the reaction products unless desired; activity can be determined for several samples simultaneously; activity can be detected for very low quantities of proteolytic enzyme; and activity against specific protein substrates can be tested using much lower amounts of the protein per assay than by the ninhydrin method or titration. Automation is also possible (15).

In these experiments, the fluorescence method was not pushed to the limits of instrument sensitivity but could easily detect 100 ng of enzyme with 100 μg of substrate. It appears that this ANS-fluorescence method may have a wide application in the detection of protein-specific proteases in various tissues, especially when the quantities of pure protein substrate obtainable are very small.

Table IV. *Comparison of Proteolytic Activity by Two Methods*

Proteolytic activity of an extract from pumpkin seeds using pumpkin seed globulin as a substrate and activity of pepsin with BSA as a substrate was determined by a fluorimetric method and a ninhydrin method. Fluorimetric activity is given as change in relative fluorescence units. Activity by the ninhydrin method is given as change in absorbance at 570 nm. Both units are comparable on the same chart paper. In both methods, the reaction volume was 3 ml.

Proteolytic System	Ninhydrin Method	Fluorimetric Method
Pumpkin seed extract-pumpkin seed globulin		
μg extract protein	530	40
units/min	0.006	0.008
units/min·mg protein	0.011	0.20
Pepsin-BSA		
μg pepsin	1	1
units/min	0.005 (I)[1]	0.02 (I)
	0.022 (II)	0.07 (II)
units/min·mg protein	5.0 (I)	20.0 (I)
	22.0 (II)	70.0 (II)

[1] (I) and (II) refer to the two rates of activity exhibited with BSA.

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